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Isolation and inheritance of microsatellite loci for the oily bittering (*Acheilognathus koreensis*): applications for analysis of genetic diversity of wild populations

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The oily bittering *Acheilognathus koreensis* is a freshwater species that is endemic to Korea and is experiencing severe declines in natural populations as a result of habitat fragmentation and water pollution. For the conservation and restoration of this species, it is necessary to assess its genetic diversity at the population level. We developed 13 polymorphic microsatellite loci that were used to analyze the genetic diversity of two populations collected from the Kum River and the Tamjin River in Korea. All loci exhibited Mendelian inheritance patterns when examined in controlled crosses. Both populations revealed high levels of variability, with the number of alleles ranging from 3 to 20 and observed and expected heterozygosities ranging from 0.500 to 0.969 and from 0.529 to 0.938, respectively. None of the loci showed significant deviation from Hardy–Weinberg equilibrium, and one pair of loci showed significant linkage disequilibrium after Bonferroni correction. Pairwise F_{ST} and genetic distance estimation showed significant differences between two populations. These results suggest that the microsatellites developed herein can be used to study the genetic diversity, population structure and conservation measure of *A. koreensis*.

Keywords: *Acheilognathus koreensis*; genetic diversity; microsatellite loci; oily bittering

Introduction

The bitterings are of the subfamily Acheilognathinae, which includes approximately 40 species and subspecies. They are a small freshwater fish with a deep body and a semi-inferior mouth. Bitterings are distributed in temperate regions of Europe and Asia, including Korea, Japan, Taiwan, and China (Banarescu 1990). The Korean bitterings are classified into two genera and 14 species, including nine endemic species (Kim et al. 2005). The oily bittering (*Acheilognathus koreensis*) used in the present study is a common freshwater fish endemic to Korea that inhabits the Kum, Seomjin, Nagdong, and Tamjin Rivers (Kim and Kim 1990; Kim and Park 2002). Natural populations of this species have recently experienced severe declines as a result of habitat fragmentation and water pollution (Kim et al. 2011). Conservation projects are currently in progress to promote increases in population size and distribution. However, knowledge of population genetic structure is fundamental for developing effective plans, and suitable DNA markers are needed for evaluation of population genetic diversity.

DNA markers such as restriction fragment length polymorphisms (RFLPs; Ikeda and Taniguchi 2002), amplified fragment length polymorphisms (AFLPs; Vos et al. 1995) and random amplified polymorphic DNA (RAPD; Williams et al. 1990) have proven useful for assessing population characteristics. Nevertheless, each of these marker systems has drawbacks. For example, RFLP analysis is arduous and requires large amounts of high-quality DNA as well as labeled probes, the AFLP technique is dependent on the dominant nature of the markers, and the RAPD approach is relatively easy to handle without any sequence prerequisites but has poor consistency and low reproducibility. Microsatellite or simple sequence repeat (SSR) markers have been used to overcome these limitations. Microsatellites are short, tandem-repeated nucleotide motifs distributed throughout the genome. Because they are highly polymorphic, easy to genotype, and co-dominantly inherited, they have been widely used in many marine species to evaluate population genetic diversity (Kim et al. 2010), construct genetic linkage maps (Kang et al. 2008), and perform pedigree analysis (McDonald et al. 2004). However, one of their limitations is the presence of null alleles. The misidentification of null alleles could lead to distortion in segregation analysis, resulting in false estimates. Thus, the Mendelian inheritance patterns for microsatellite loci should be determined in controlled parent–offspring lines prior to using them in population or parentage studies. The other limiting factor is

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that these markers must be specifically developed for the species of interest, necessitating an initial cost associated with their identification, given the requirement for sequence information. However, once developed, these markers are easy to use, highly reproducible, and suitable for multiplexing. However, no microsatellite markers have been developed for *A. koreensis* to date. Therefore, we developed a set of polymorphic microsatellite markers and assessed their inheritance modes in the three *A. koreensis* families. We also examined their genetic diversity in natural populations to assess their utility as genetic markers.

Materials and methods

Sample collection and DNA isolation

A total of 58 individuals of oily bittering (*A. koreensis*) were collected from the Kum River ($N = 26$) and Tamjin River ($N = 32$), Korea. Genomic DNA was extracted from the caudal fins using the TNES-urea buffer method (Asahida et al. 1996).

Production of A. koreensis *families*

A. koreensis families were produced by single-pair matings with *A. koreensis* adults caught from the Deokcheon River in 2010 at the Biotechnology Research Division of the National Fisheries Research and Development Institute (NFRDI), Korea. Three females (F1–F3) and three males (M1–M3) were crossed in different combinations, and three families were successfully obtained (family A, M1xF1; family B, M2xF2; family C, M3xF3). A total of 30 juveniles were randomly sampled from each family when they were approximately 2 months old. The whole body of the juvenile was used to isolate genomic DNA.

Isolation of microsatellites

A partial genomic library enriched for GT repeats was constructed by slightly modifying the procedure described by Hamilton et al. (1999). Specifically, genomic DNA extracted from an adult *A. koreensis* was digested with the enzymes *Alu* I and *Rsa* I (New England Biolabs), and DNA fragments ranging from 200 to 800 bp were isolated and ligated to SNX/SNX rev linker sequences. Linker-ligated DNA was amplified using SNX as a polymerase chain reaction (PCR) primer, and PCR products were hybridized to biotinylated $(GT)_{10}$ probes attached to streptavidin-coated magnetic beads (Promega). Following elution from the beads, the repeat-enriched DNA was made double stranded and amplified using SNX linker as a primer. The amplified DNA was digested with the enzyme *Nhe* I and ligated into a *Xba* I-digested pUC18 vector, followed by transformation into *Escherichia coli* DH5α competent cells. Positive clones with repeats were identified by PCR with $(GT)_{10}$ and M13 primers. A negative control with no template was included in each PCR. The PCR products were analyzed on 1.5% agarose gels, and the clones producing two or more bands were considered to contain a microsatellite locus. Plasmid DNA of positive clones was purified using an Acroprep 96-well filter plates (PALL). The plasmids were sequenced using the Big Dye Terminator reaction kit on the ABI 3130*xl* automated sequencer (Applied Biosystems).

Primer design

Primers were designed from the unique sequences flanking microsatellite motifs using the OLIGO 5.0 software (National Biosciences). PCR was performed using DNA samples originally used for microsatellite isolation to establish whether the desired size product was amplified. PCR reactions were performed in 15-μL reaction volumes containing 50 ng of genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, and 0.5 U of Taq DNA polymerase. All amplifications were performed on a PTC-220 thermal cycler (MJ Research). The amplification was carried out under the following conditions: an initial denaturation at 95°C for 15 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at temperatures ranging from 45°C to 60°C, and extension at 72°C for 30 s, and then a final extension at 72°C for 15 min. The PCR products (>100 bp) were analyzed on 1.5% agarose gels. Small-size PCR products (<100 bp) were analyzed in the 3% metaphor agarose gel (BioProduct).

Genotyping

A total of 13 microsatellite loci were used to genotype the 58 *A. koreensis* individuals from two populations. For fluorescent detection of the PCR products, the forward primer in each pair was end-labeled commercially with the dyes 6FAM, NED, or HEX (Applied Biosystems). PCR reactions performed in 10-μL volumes containing 10 ng of genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 3 pmol of each primer, 0.5 × Band Doctor, and 0.5 U of f-Taq DNA polymerase (Solgent). A PCR cycle was used as described above at the primer-specific annealing temperature (Table 1). The lengths of the PCR products were determined with an ABI 3130*xl* Genetic Analyzer (Applied Biosystems) using the GeneScan-400HD (ROX) size standard (Applied Biosystems) and

Table 1. Thirteen microsatellite loci from *Acheilognathus koreensis* and their amplification information.

Locus	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Length (bp)	GenBank accession no.
Ak64	$(AC)_{13}$	F: 6FAM-GCCTGCTCTCGTGGTTACGC R: TGTCATGATGAACCACGATGCTC	60	89	JN315693
Ak110	$(AC)_9$	F: HEX-AGATGTAAAAAGTGCCCATGTGTC R: AAGAAAAGAGGGTTGTGAGGTCA	55	138	JN315694
Ak154	$(AC)_{37}$	F: NED-AGCACAAGAATTACACATCACCT R: CGTGACAAAACATGGAAACA	58	157	JN315695
Ak181	$(TC)_{29}$	F: HEX-CTGACTCGATCAAGAGCATAAAT R: GAAGCACAAGGAACAATACTGAG	55	154	JN315696
Ak382	$(TC)_{22}$	F: 6FAM-CAGCCATTGGAAGCGGTTAT R: AACGGATGTGTGGAGGTAGATTG	60	104	JN315697
Ak424	$(TC)_6(AC)_{15}$	F: 6FAM-GAGTGATCGCAGCTAAATTAGAG R: ATCATAACCCTAATGCCATACAG	58	107	JN315698
Ak455	$(GT)_{11}$	F: 6FAM-CACTGCTGAGTCTGAGCTTTTAT R: GGTTAAAGGTGTAATTACCAGCC	58	108	JN315699
Ak462	$(GA)_{17}$	F: NED-TGCAAAGTCAGACAAGAGTTATC R: CTCTGACGTAGTGCTGCTG	58	171	JN315700
Ak468	$(AC)_{13}$	F: NED-CAGCACAATGACAGTCTACCAAG R: ATCCCCAGGTGAGAGTCGT	58	162	JN315701
Ak474	$(AC)_{13}$	F: 6FAM-CATGCATGTCCTCCTGTGTG R: TTACAAGCAACCAAACGAGAGAA	58	108	JN315702
Ak479	$(AC)_{16}$	F: HEX-GAGGTCTGGGAATCATCAAAAC R: GTTGCTATGGATACCGTCTGTCT	58	125	JN315703
Ak625	$(TC)_{12}(AC)_9$	F: HEX-GAGAAGCAGATGAGAATACACACT R: ACCAACATCACCCTAAAATACC	60	126	JN315704
Ak686	$(AC)_6 \ldots (AC)_9$	F: HEX-AGCGCATCGGAGAGGAGTGCTC R: TGCGGGGTCCTCTGAGATGTGTT	60	156	JN315705

…, GCATACACACAAGCACGC; F, forward primer; R, reverse primer; T_a, optimal annealing temperature.

analyzed using the software GeneScan 3.7 and Genotyper 3.7 (Applied Biosystems).

Data analysis

The number of alleles, allelic frequencies, polymorphism information contents (PICs), and the observed and expected heterozygosities at each locus in each population were calculated using the CERVUS 3.0 program (Marshall et al. 1998). Deviations from Hardy–Weinberg equilibrium (HWE) for each population at each locus using a Markov-chain algorithm (Guo and Thompson 1992) and linkage disequilibrium of all pairs of loci were tested using the GENEPOP 3.4 software (Raymond and Rousset 1995). We also estimated F_{IS} values (Weir and Cockerham 1984), which can determine HWE departures within a population. Genetic distances between populations were estimated using the PHYLIP software package version 3.68 (Felsenstein 1989) with (Ds) standard genetic distance (Nei 1972). F_{ST} was estimated using an ARLEQUIN software package version 1.1 (Schneider et al. 1997). The mode of inheritance and presence of null alleles at 13 microsatellite loci was evaluated using DNA from parents and 30 offspring from each of three families. To ascertain whether alleles are inherited in a Mendelian fashion, observed genotypic ratios in offspring to Mendelian expectations (1:1, 1:2:1, and 1:1:1:1) were analyzed using a χ^2 goodness-of-fit test.

Results

Development of A. koreensis *microsatellite markers*

From approximately 1200 genomic library clones examined, 800 clones with inserts were randomly selected and screened for the repeat using PCR, which yielding 576 (72%) true positive clones. These were sequenced, producing a total of 463 (58%) sequences containing SSRs, of which 276 (35%) were eliminated because they possessed the no flanking sequences. A total of 187 (23%) sequences containing microsatellites were finally obtained, and 136/187 (72.7%) of the sequences contained AC/GT microsatellites, while 51/187 (27.3%) were found to contain TC/GA microsatellites. Among the 187 microsatellites, 122 were perfect (65.2%), 10 (5.4%) were imperfect, and 55 (29.2%) were classified as compound. A total of 92 primer pairs from the 187 sequences were designed to amplify microsatellite-containing regions of the genome; the other 95 sequences were abandoned because they contained

inserts that were too small or large (33/95) or contained repeat motifs that were too proximate to the cloning site (62/95). The numbers of repeats in these sequences ranged from 3 to 105 repeat units. Only 36 (39%) of the 92 primer pairs tested successfully amplified the target region, and the remaining pairs either failed to amplify or produced nonspecific bands. Finally, we labeled 24 (26%) of the 36 primer pairs with fluorescence dyes because they exhibited polymorphisms in 10 different *A. koreensis* individuals when PCR products were analyzed by agarose/metaphor-gel electrophoresis. However, when PCR products were genotyped, 11/24 (46%) were not scorable due to excessive stutter, apparent amplification of multiple loci, and/or failure to amplify DNA from a large number of individuals despite attempts at reoptimization. The repeat motif, product size, and annealing temperatures at each of 13/24 (54%) microsatellite loci are presented in Table 1. Representative sequences for all 13 of the microsatellite regions were deposited into GenBank under the accession numbers JN315693–JN315705 (Table 1).

Inheritance of A. koreensis *microsatellite loci*

The inheritance and segregation patterns for 13 microsatellite loci in the parents and offspring of three families are presented in Table 2. The various genotypic combinations of mated males and females produced three different segregation ratios: 1:1, 1:2:1, and 1:1:1:1. Of the 39 genotypic ratios examined (13 loci $\times$ 3 families), 38 genotypic frequencies (97%) were in accordance with Mendelian expectations. For example, family C at the locus Ak64 produced the expected segregation of 1:1:1:1 ratio given that the male and female parents were heterozygous for 83/89 and 89/107 genotypes, respectively (Table 2). The offspring exhibited four genotypes (83/89, 83/107, 89/89, and 89/107) that segregated according to expectation ($\chi^2 = 1.20$, $df = 3$). Family C at the locus Ak424 exhibited distorted segregation ratios ($p < 0.05$) and had an excess of heterozygotes (101/105). Additionally, family A at locus Ak154 had both male and female parents with a single homozygous genotype with the same allele size (99). Thus, all offspring genotypes were identical to both parents.

Genetic diversity in A. koreensis *populations*

Table 3 summarizes the genetic diversity of each population at each locus. The levels of genetic diversity varied depending on the locus. All loci were successfully amplified in two populations and found to be polymorphic. A total of 182 alleles were detected at 13 loci analyzed in 58 individuals of oily bittering. The number of alleles per locus varied from 6 (Ak110) to 27 (Ak181), with a mean value of 14.0 in the total population. The number of alleles ranged from 3 to 16, with a mean of 8.54 per locus in the Kum River population and 4 to 20 with a mean of 9.23 in the Tamjin River population. The mean number of alleles per locus was similar in the two populations, but there were marked differences for locus Ak154, which exhibited 15 alleles from the Tamjin River and only seven alleles from the Kum River. The mean number of shared alleles between two populations was 3.77. An average of 26.9% of the alleles detected was shared between the two populations.

Measures of genetic variation for each population (PIC, and observed and expected heterozygosity) are given in Table 3. In the Kum River, the observed heterozygosity ranged from 0.500 to 0.962 (mean = 0.766), and the expected heterozygosity ranged from 0.529 to 0.938 (mean = 0.771). In the Tamjin River, the observed heterozygosity ranged from 0.563 to 0.969 (mean = 0.738), and the expected heterozygosity ranged from 0.516 to 0.927 (mean = 0.749). Although it varied among loci, the mean observed heterozygosity was lower than the mean expected heterozygosity for both populations. None of the loci showed significant deviation from HWE ($p > 0.01$). One pair of loci (Ak64 and Ak181) showed significant linkage disequilibrium after Bonferroni correction at the total population level ($p < 0.004$). The PIC of oily bittering from the Kum River (0.719) was higher than that of oily bittering from the Tamjin River (0.707). All loci were tested for a inbreeding coefficient (F_{IS}), which was positive for three loci in two populations, indicating a deficit of heterozygotes, whereas for five loci, the parameter was negative, indicating an excess of heterozygous genotypes. A broad range in F_{IS} index values was found among the loci, ranging from –0.171 (Ak625 in the Tamjin River) to 0.237 (Ak468 in the Kum River). The overall F_{IS} value among all loci was higher than zero, indicating some level of heterozygote deficiency. The Nei standard genetic distance based on allele frequencies between two populations was 1.51. The genetic differentiation (F_{ST}) between the two populations was 0.195, which was statistically significant ($p < 0.01$).

Discussion

Microsatellites have been widely used as genetic markers in many marine organisms including flounder, oyster, and shrimp because of their high degree of variability, which makes them powerful tools for population genetic analyses (Carlsson et al. 2006; Liu et al. 2006; Kim et al. 2010). They have also been used for freshwater fish such as *Cambaroides similis* (Ahn et al. 2011), *Brycon opalinus* (Barrosoa et al. 2005), and

Table 2. Inheritance of 13 microsatellite loci from three *Acheilognathus koreensis* families produced by controlled crosses.

Locus	Family	Parental genotype	N[a]	F1 offspring genotype: Observed number (expected number[b])				χ^2
Ak64	A	♂89/91	30	89/89	89/91			0.53
		♀89/89		17(15)	13(15)			
	B	♂89/89	30	83/89	89/89			0.13
		♀83/89		14(15)	16(15)			
	C	♂83/89	30	83/89	83/107′	89/89	89/107	1.20
		♀89/107		8(7.5)	9(7.5)	8(7.5)	5(7.5)	
Ak110	A	♂134/136	30	132/134	132/136			0.00
		♀132/132		15(15)	15(15)			
	B	♂132/136	30	132/132	132/136	136/136		0.40
		♀132/136		6(7.5)	16(15)	8(7.5)		
	C	♂134/136	30	132/134	134/136	132/136	136/136	2.27
		♀132/136		6(7.5)	9(7.5)	10(7.5)	5(7.5)	
Ak154	A	♂99/99	30	99/99				-[d]
		♀99/99		30(30)				
	B	♂109/119	30	101/109	109/109	101/119	109/119	3.33
		♀101/109		7(7.5)	11(7.5)	4(7.5)	8(7.5)	
	C	♂103/109	30	101/103	101/109			0.53
		♀101/101		17(15)	13(15)			
Ak181	A	♂122/160	30	122/148	122/158	148/160	158/160	1.47
		♀148/158		9(7.5)	7(7.5)	9(7.5)	5(7.5)	
	B	♂100/100	30	100/122	100/160			0.13
		♀122/160		16(15)	14(15)			
	C	♂124/160	30	120/124	124/140	120/160	140/160	1.73
		♀120/140		5(7.5)	7(7.5)	10(7.5)	8(7.5)	
Ak382	A	♂75/77	30	75/75	75/77	77/77		0.40
		♀75/77		9(7.5)	14(15)	7(7.5)		
	B	♂85/101	30	75/85	77/85	75/101	77/101	0.13
		♀75/77		8(7.5)	8(7.5)	7(7.5)	7(7.5)	
	C	♂75/85	30	75/77	75/91	77/85	85/91	1.20
		♀77/91		8(7.5)	5(7.5)	8(7.5)	9(7.5)	
Ak424	A	♂95/105	30	95/95	95/105	105/105		1.13
		♀95/105		8(7.5)	17(15)	5(7.5)		
	B	♂95/105	30	95/105	95/107	105/105	105/107	2.53
		♀105/107		11(7.5)	7(7.5)	7(7.5)	5(7.5)	
	C	♂101/105	30	97/101	101/105	97/105	105/105	10.3[c]
		♀97/105		4(7.5)	15(7.5)	6(7.5)	5(7.5)	

Locus	Family	Parental genotype	N	F1 offspring genotype: Observed number (expected number)				χ^2
Ak462	A	♂157/167	30	157/157	157/167	167/167		0.60
		♀157/167		6(7.5)	15(15)	9(7.5)		
	B	♂155/171	30	155/155	155/173	155/171	171/173	1.73
		♀155/173		8(7.5)	5(7.5)	10(7.5)	7(7.5)	
	C	♂157/167	30	155/157	157/171	155/167	167/171	3.33
		♀155/171		10(7.5)	5(7.5)	5(7.5)	10(7.5)	
Ak468	A	♂153/163	30	153/153	153/163	163/163		1.20
		♀153/163		5(7.5)	16(15)	9(7.5)		
	B	♂153/163	30	147/153	147/163			0.53
		♀147/147		17(15)	13(15)			
	C	♂153/159	30	153/153	153/163	153/159	159/163	0.67
		♀153/163		6(7.5)	9(7.5)	7(7.5)	8(7.5)	
Ak474	A	♂99/101	30	99/105	99/115	101/105	101/115	2.80
		♀105/115		5(7.5)	11(7.5)	6(7.5)	8(7.5)	
	B	♂101/113	30	101/105	101/115	105/113	113/115	2.53
		♀105/115		7(7.5)	11(7.5)	5(7.5)	7(7.5)	
	C	♂99/101	30	99/99	99/101	101/101		0.60
		♀99/101		6(7.5)	15(15)	9(7.5)		
Ak479	A	♂121/141	30	109/121	121/121	109/141	121/141	0.13
		♀109/121		8(7.5)	7(7.5)	7(7.5)	8(7.5)	
	B	♂121/141	30	109/121	113/121	109/141	113/141	2.27
		♀109/113		11(7.5)	6(7.5)	6(7.5)	7(7.5)	
	C	♂109/121	30	109/121	109/137	121/121	121/137	1.47
		♀121/137		10(7.5)	6(7.5)	8(7.5)	6(7.5)	
Ak625	A	♂115/125	30	115/123	115/127	123/125	125/127	5.47
		♀123/127		4(7.5)	5(7.5)	12(7.5)	9(7.5)	
	B	♂125/127	30	115/125	125/125	115/127	125/127	2.27
		♀115/125		6(7.5)	11(7.5)	6(7.5)	7(7.5)	
	C	♂125/127	30	125/125	125/127	127/127		0.40
		♀125/127		6(7.5)	16(7.5)	8(7.5)		
Ak686	A	♂151/153	30	151/153	153/153			2.13
		♀153/153		19(15)	11(15)			
	B	♂151/153	30	145/151	151/151	145/153	151/153	4.90
		♀145/151		10(7.5)	11(7.5)	4(7.5)	5(7.5)	
	C	♂145/151	30	145/145	145/153	145/151	151/153	1.20
		♀145/153		8(7.5)	9(7.5)	8(7.5)	5(7.5)	

Table 2 (*Continued*)

Locus	Family	Parental genotype	N^a	F1 offspring genotype: Observed number (expected number[b])			χ^2	Locus	Family	Parental genotype	N	F1 offspring genotype: Observed number (expected number)	χ^2
Ak455	A	♂108/122	30	108/122	122/122		1.20						
		♀122/122		12(15)	18(15)								
	B	♂102/108	30	102/106	102/108	106/108	108/108	2.53					
		♀106/108		7(7.5)	5(7.5)	7(7.5)	11(7.5)						
	C	♂102/106	30	102/106	102/108	106/106	106/108	1.20					
		♀106/108		8(7.5)	5(7.5)	9(7.5)	8(7.5)						

[a]N is the number of offspring scored at each locus.
[b]Mendelian expectations in each genotypic class are shown in parentheses.
[c]Nominal significant deviation from Mendelian ratio ($p < 0.05$). For $p < 0.05$, critical χ^2 value is 7.82 for df (degree of freedom) = 3, 5.99 for df = 2, 3.84 for df = 1.
[d]Chi-square analysis was not performed since both parents possessed homozygous genotypes.

Chondrostoma lusitanicum (Sousa et al. 2008). However, until now, no information was available on the use of microsatellite markers for oily bittering (*A. koreensis*). Here, we isolated and characterized the first microsatellite markers in this species and analyzed the population genetic diversity of two wild populations.

The conventional protocols for isolating microsatellites are cost, time, and labor intensive, and the efficiency of microsatellite isolation is low, ranging from 0.045% to 12% (Zane et al. 2002). To overcome these challenges, several enrichment techniques have been developed, which are based on the principal of capturing microsatellites from genomic DNA by hybridization with synthetic oligonucleotides bound to Nylon membranes or magnetic particles (Zane et al. 2002). We constructed a microsatellite enrichment library for the oily bittering using $(GT)_{10}$ biotin-labeled probes, and 80% (463/576) of the positive clones contained microsatellite repeats. This efficiency is lower than that in tilapia (96%; Carleton et al. 2002) but higher than that in cutlassfish (48%; An et al. 2010). A total of 72.7% of sequences contained AC/GT microsatellites in accordance with the GT probes, and 27.3% contained TC/GA microsatellites, which were randomly obtained without using any GA probes. This indicated that $(AC)_n$ and $(TC)_n$ repeats are widely distributed throughout the oily bittering genome. The close proximity of the repeat motifs to the cloning sites yielded possible primers for only 66.8% of the microsatellites sequenced. This issue is common for both conventional and microsatellite-enriched genomic libraries (Alghanim and Almirall 2003).

It is common for microsatellite loci to exhibit null alleles (Banks et al. 1999; Jones et al. 1999), which were found in up to 25% of loci examined in humans (Callen et al. 1993). No obvious pattern has been observed for the occurrence of null alleles, so without data from controlled crosses, it is not possible to estimate null allele frequencies in a wild population. Thus, an inheritance study of microsatellite loci is a fundamental prerequisite for using molecular markers in genetic studies. Previous studies have emphasized the importance of testing for the inheritance of potential genetic markers against known parent–offspring relationships (Jerry et al. 2004), and we found this process very valuable for detecting null alleles in oily bittering. With the exception of the Ak424 locus in family C, all 13 loci followed Mendelian inheritance patterns. It is reasonable to assume that this deviation in a single family at one locus may be caused by random variation. The incidence of segregation distortion (2.6%) that we observed was slightly lower than that reported in Japanese flounder (3.6%, Sekino and Hara 2001). Some authors have recommended that loci with null alleles should be excluded from parentage analyses

Table 3. Genetic diversity parameters for the two *A. koreensis* populations.

	No. of alleles				Kum River ($N=26$)				Tamjin River ($N=32$)			
	Total	Shared	N_A	H_O	$H_E(P)$	PIC	F_{IS}	N_A	H_O	$H_E(P)$	PIC	F_{IS}
Ak64	12	2	7	0.846	0.790(0.256)	0.748	−0.072	7	0.844	0.793(0.668)	0.747	−0.066
Ak110	6	1	3	0.500	0.529(0.844)	0.403	0.055	4	0.594	0.615(0.896)	0.534	0.034
Ak154	20	2	7	0.654	0.789(0.161)	0.740	0.174	15	0.719	0.820(0.096)	0.788	0.125
Ak181	27	7	14	0.885	0.882(0.154)	0.852	−0.003	20	0.969	0.927(0.689)	0.906	−0.046
Ak382	19	7	16	0.962	0.938(0.081)	0.914	−0.025	10	0.813	0.855(0.471)	0.824	0.050
Ak424	10	3	7	0.769	0.728(0.020)	0.668	−0.058	6	0.719	0.753(0.371)	0.698	0.047
Ak455	9	5	8	0.692	0.685(0.855)	0.638	−0.011	6	0.750	0.743(0.616)	0.687	−0.010
Ak462	19	5	12	0.885	0.851(0.453)	0.816	−0.041	12	0.781	0.876(0.193)	0.848	0.110
Ak468	15	2	7	0.577	0.753(0.085)	0.698	0.237	10	0.594	0.611(0.630)	0.581	0.029
Ak474	13	6	11	0.769	0.867(0.037)	0.835	0.115	8	0.563	0.516(0.897)	0.489	−0.092
Ak479	16	4	9	0.885	0.811(0.695)	0.774	−0.092	11	0.750	0.845(0.685)	0.813	0.114
Ak625	9	3	7	0.885	0.811(0.667)	0.767	−0.092	5	0.719	0.616(0.668)	0.554	−0.171
Ak686	7	2	3	0.654	0.588(0.259)	0.497	−0.114	6	0.781	0.763(0.270)	0.714	−0.025
Mean	14	3.77	8.54	0.766	0.771(0.033)	0.719	0.006	9.23	0.738	0.749(0.813)	0.707	0.008

N, sample size; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphism information content; F_{IS}, population inbreeding coefficient. P is the value estimated by the Fisher's exact test in the Markov-chain method.

(Pemberton et al. 1995). The results obtained here indicated that these loci could be useful for parentage analysis, population genetic studies, and genetic mapping in *A. koreensis*.

Gene heterozygosity, also referred to as gene diversity, is a suitable parameter for investigating genetic variation. For a marker to be useful for measuring genetic variation, it should have a heterozygosity of at least 0.3 (Takezaki and Nei 1996). The range of expected heterozygosity of the markers in the two populations analyzed here was between 0.529 and 0.938; thus, the markers were appropriate for measuring genetic variation. PIC value is related to the availability and utilization efficiency of a marker; the higher the PIC value of the marker is in a population, the higher the heterozygote frequency is and the more genetic information it provides (Arora et al. 2004). Genetic markers showing PIC values higher than 0.5 are normally considered informative for population genetic analyses (Botstein et al. 1980). In this study, all of the 13 microsatellite loci were highly polymorphic. The mean PIC value across all loci exceeded 0.5, which could provide sufficient information for the assessment of genetic diversity.

The F_{ST} value is a useful measure for determining genetic differentiation among populations, with different values indicating different degrees of variation. An F_{ST} value within the range of 0.05–0.15 indicates moderate differentiation, whereas 0.15–0.25 suggests substantial differentiation, and >0.25 indicates very high differentiation (Wright 1978). In this study, the F_{ST} value was 0.195 between the Kum River and Tamjin River populations, indicating that there was a substantial differentiation between two populations. It was clear that 19.5% of total genetic variation corresponded to differences at the population level, and the remaining 80.5% was the result of differences among individuals. The population genetic distance calculated by Nei's standard method was 1.51, also indicating high differentiation between the two populations.

Conclusion

In the present study, we developed the first set of microsatellite loci for *A. koreensis*. The microsatellite markers were polymorphic within populations and between populations. We provided a preliminary estimate of genetic diversity and population differentiation of the oily bittering. Moreover, these markers will be a useful for the study of population genetic structure and establishment of effective conservation strategies. We are currently performing analyses to determine the fine-scale structuring of oily bittering populations in Korea.

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